INFLUENCE OF CARBONIC ANHYDRASE INHIBITORS ON SHELL GROWTH OF A FRESH-WATER SNAIL, PHYSA HETEROSTROPHA [1]

JOHN A. FREEMAN

Department of Biology, Winthrop College, Rock Hill, S. C.

The enzyme carbonic anhydrase catalyses reversibly the reaction

$$CO_2 + H_2O \rightarrow H_2CO_3$$

(Berliner and Orloff, 1956) and has been shown to accelerate both deposition and dissolution of calcium carbonate *in vitro* (Meldrum and Roughton, 1933). In view of its possible implication in shell formation, the mantle tissues of molluscs have been examined and the enzyme demonstrated in several of them (Florkin and de Marchin, 1941; Maetz, 1946; Freeman and Wilbur, 1948; Wilbur and Anderson, 1950; Stolkowski, 1951; Tsujii and Machida, 1953; Ikinago, 1954; Kawai, 1954a, 1954b; Larraneta and Ponz, 1954; Clark, 1957). However, among 20 species examined, Freeman and Wilbur (1948) found little or no carbonic anhydrase activity in mantle tissue in two, a pelecypod and a gastropod, both shell-formers, and Stolkowski (1951) found none in the mantles of *Ostrea edulis* and several other shell-forming molluscs. Such results suggest that at least in some species the enzyme is not essential for shell formation.

Stolkowski (1951) apparently first used a carbonic anhydrase inhibitor, benzenesulfonamide, in studies of the role of the enzyme in molluscs, observing decreased shell regeneration in *Helix aspersa* in the presence of the drug. He concluded that the enzyme is important in determining the crystalline form in which calcium carbonate appears in the shell. Wilbur and Jodrey (1955) found that two carbonic anhydrase inhibitors, 2-benzothiazolesulfonamide and Diamox (2-acetylamino-1,3,4-thiadiazole-5-sulfonamide), markedly decrease the rate of deposit of Ca^{45} in the shell of *Crassostrea virginica*. Abolins-Krogis (1958) in a study of shell regeneration in *Helix pomatia* concludes on the basis of as yet unpublished data on carbonic anhydrase inhibition that (p. 36) the enzyme "is necessary for the formation of calcium carbonate crystals in the regenerating shell." These studies suggest a role of carbonic anhydrase in shell formation but are inconclusive in establishing it. The two inhibitors used by Wilbur and Jodrey, 2-benzothiazolesulfonamide and Diamox, exhibited quite different extents of inhibition of Ca^{45} deposition in the intact animal, only Diamox affecting deposition in a manner consistent with the hypothesis that the results were due to carbonic anhydrase inhibition. Even though in the mantle-shell preparation used in part of the studies the two drugs had effects consistent with the idea that the enzyme is important in determining the rate of calcium deposit, the side effects of one of the drugs suggest caution in interpretation of the data. Further, the

[1] This study has been supported in part by a grant from the Southern Fellowships Fund.

data are consistent with another interpretation, that carbonic anhydrase influences the exchange of calcium between the shell and the extrapallial fluid. This is an exchange which may not include the reactions which serve as a pathway for shell formation.

The study of Abolins-Krogis (1958) indicates that calcium carbonate crystals did not develop in the presence of a carbonic anhydrase inhibitor. Since the reaction catalyzed by the enzyme occurs at a very appreciable rate, proceeding 90 per cent to equilibrium in 200 seconds (Berliner and Orloff, 1956), it would be expected that during inhibition of the enzyme deposition of calcium carbonate would occur at a decreased rate but would not cease.

One method of resolving the dilemma would be to study the growth of shell in the presence of a number of carbonic anhydrase inhibitors, for if a series of side effects were operative among the drugs in decreasing shell development these effects should show different extents or at least statistically modify in different ways the pattern for the growth of the populations treated with different drugs. On the other hand, if the effect on shell development is statistically similar in the case of several known carbonic anhydrase inhibitors this would strengthen the evidence for a direct role of the enzyme in shell development. In the present study the hypothesis that carbonic anhydrase is important in molluscan shell development has been tested by a study of the influence of five carbonic anhydrase inhibitors on the growth of the shell of the pond snail, *Physa heterostropha*.[2]

Methods

Adult snails of a local population were maintained as stock cultures on lettuce with oyster shell chips in the bottom of aquaria. Young snails not over a month old were removed from the stock aquaria as required and placed in 4-inch fingerbowls containing 100 ml. water which had been equilibrated with precipitated calcium carbonate for at least a week. Three or four snails from about 2 to 5 mm. in length, which were individually distinguished in the course of measurements, were placed in each fingerbowl along with lettuce. Twenty-four hours later each snail was measured, using a micrometer calibrated to 0.01 mm., and the water was replaced. At this time appropriate volumes of solutions of the drugs (made up in the $CaCO_3$-saturated water) were substituted for a part of the water in dishes containing the experimental animals. Subsequently the water or inhibitor solution was replaced daily and lettuce was added as needed. (For information on feeding, see Table I.) The snails were measured on alternate days for ten days. In the few cases in which snails were lost, died or were accidentally killed, the measurements were included if data were available for six or more days of growth. (Such losses were: 8 snails died during the major series of observations, 6 were killed accidentally during handling, and there were 3 losses which were unaccounted for among 672 snails.)

The experiment was carried out in the room in which the snails were reared, at a temperature which in most experiments approximated 26° C. (In a preliminary study the temperature approximated 21°, as indicated in appropriate places in the tables.)

[2] The author appreciates the identification of the snail by Dr. H. Van der Schalie of the University of Michigan.

TABLE I

Growth of Physa heterostropha fed in three ways. Data for snails having median lengths during growth periods of 3.00–5.99 mm.

Feeding	No. snails	Growth (mm./day)		
		Median	Mean	Range
I	12	0.11	0.10 ± 0.015	0.05–0.13
II	55	0.25	0.27 ± 0.010	0.07–0.38
III	30	0.40	0.41 ± 0.014	0.28–0.49

Observations were at *ca.* 26°. Fresh lettuce was available to all snails continuously, the amount of decaying lettuce varying. In feeding method I, each morning any piece of lettuce showing signs of decay was removed and replaced with fresh lettuce. In method II, each piece of lettuce was left until all parts showed signs of decay. Then it was removed and replaced with fresh lettuce. In method III, decaying lettuce was available at all times and decaying pieces were removed when only plant fibers remained.

The inhibitors used were Diamox (2A),[3] 2-benzothiazolesulfanilamide (2B)[3] (Miller, Dessert and Roblin, 1950), benzenesulfonamide (BS), p-toluenesulfonamide (TS) (Krebs, 1948) and sulfanilamide (SA) (Mann and Keilin, 1940).

RESULTS

Examination of preliminary data in graphs indicated approximately uniform growth rates for snails having median lengths ranging from 2 to 6 mm. during growth periods and lower rates for those having lengths outside this range. More critical examination of the data and subsequently of data for all control groups, using the Kruskal-Wallis test (Kruskal and Wallis, 1952), indicates the range of lengths having statistically uniform growth rates is more restricted, from 3.00 to 5.99 mm. when data are examined in classes having 0.50 mm. ranges. All data for controls have been examined and, with the exception of a single class among those subjected to feeding method I, show no statistically significant variations in growth rates among classes having median lengths of 3.00 to 5.99 mm. within any method of feeding at a given temperature. As determined by the Kruskal-Wallis test, probability (p) for controls ranges from 0.3 to 0.8, with the exception indicated. Statistically significant variations among classes within experiments were obtained in each case in which a broader range of median lengths was used. Inclusion of all data in calculations would have made no difference in the over-all picture, for among the control experiments the mean growth rates of snails subjected to different feeding regimes would have been 0.10, 0.24 and 0.37 instead of 0.10, 0.25 and 0.41, and similar differences were found for experimental populations. The difference is in part determined by the proportion of measurement made on snails outside the range 3.00 to 5.99 mm.

Preliminary experiments indicated that the manner of feeding greatly influenced the rate of growth. Although fresh lettuce supported slow growth, the most rapid growth was obtained only if decaying lettuce was available to the

[3] Diamox and 2-benzothiazolesulfanilamide were supplied by Dr. Emmanuel Waletzky of American Cyanamid Co.

TABLE II

Growth of Physa heterostropha *in 2-benzothiazolesulfonamide. (Data for periods in which median lengths were 3.00–5.99 mm.)*

Feeding	Temp. (°C.)	Conc. 2B (mg./l.)	No. snails	Growth (mm./day)		
				Median	Mean	Range
I	21	0.0	12	0.11	0.10 ± 0.015	0.05–0.13
		0.1	12	0.12	0.11 ± 0.020	0.08–0.19
		0.5	12	0.10	0.12 ± 0.018	0.01–0.28
		1.0	12	0.01	0.02 ± 0.005	0.00–0.08
II	26	0.0	55	0.25	0.27 ± 0.010	0.07–0.38
		0.1	17	0.21	0.22 ± 0.013	0.12–0.30
		0.2	15	0.17	0.18 ± 0.013	0.06–0.26
		0.5	49	0.05	0.07 ± 0.005	0.00–0.14
		1.0	14	0.00	0.00 ± 0.001	0.00–0.02

snails at all times. The rate of growth of snails used in control experiments at 26° has been compared for three feeding methods with the results indicated in Table I.

In all experiments subsequently presented in this report feeding was by method III which gave most rapid growth, unless otherwise indicated, and data are for growth periods during which the median lengths of the snails were from 3.00 to 5.99 mm.

The effect of 2B on growth was quite different from that of the other four drugs and so is presented separately in Table II. Additional information on the effects of 2B were obtained in preliminary experiments conducted at *ca.* 21°. In these experiments, of 27 snails exposed to concentrations of 2B from 2.0 to 10.0

TABLE III

Comparison of growth rates of Physa heterostropha *treated with concentrations of 2A, TS, BS and SA showing maximum inhibition of growth with untreated controls. Data for periods in which median lengths of snails were within the range 3.00–5.99 mm.*

Feeding	Temp.* (°C.)	Drug	Drug conc. (mg./l.)	No. snails	Mean growth (mm./day)	Per cent inhibition	p**
III	21	none	0.0	16	0.23 ± 0.014		
		2A	0.5–15.0	25	0.15 ± 0.009	35	<0.00003
II	26	none	0.0	55	0.27 ± 0.010		
		2A	2.0–5.0	65	0.15 ± 0.007	44	0.0013
III	26	none	0.0	30	0.41 ± 0.014		
		BS	50	15	0.26 ± 0.014	37	
		TS	50	16	0.26 ± 0.016	37	<0.00003
		SA	10.0–50	30	0.28 ± 0.015	32	

* Temperatures varied within approximately two degree ranges.

** Values for p have been determined using the Mann-Whitney U test for comparing the data for control and experimental groups (Mann and Whitney, 1947).

mg. per liter, 24 died within two days and the remaining three within four days. At concentrations of 0.5 and 1.0 mg. per liter, *ca.* 50% mortality occurred within four days. One of six died between the tenth and twelfth day in 0.25 PPM 2B and none of 15 maintained as controls or in 0.05 PPM 2B died within 12 days. Mortality was lower at 26°, 4 of 87 snails dying within 10 days in 2B solutions of concentrations 0.5 and 1.0 mg. per liter. Among 55 controls and 32 snails in 0.1 or 0.2 PPM 2B, none died within 10 days.

The four remaining drugs when used with snails growing at the more rapid rates (feeding methods II and III) showed maximum inhibition of growth to comparable extents. The results of these experiments are presented in Table III. The data for concentrations showing maximum inhibition have been examined in classes (as indicated above for control experiments) using the Kruskal-Wallis test and found to be statistically homogeneous (p ranges from 0.1 to 0.5). Data for BS, TS and SA obtained in simultaneous observations have been similarly compared as a group with their controls. The value of p thus obtained was < 0.00003.

A series of experiments using Diamox was run to compare inhibition of growth of slowly growing snails with that of rapidly growing ones. Mean growth rates

TABLE IV

Growth rates of snails subjected to various concentrations of Diamox and fed for slow growth (Feeding I) at 26° C.

Conc. Diamox	0.0	0.1	0.2	0.3	0.4	0.5	1.0	1.5
Mean growth rate (mm./day)	0.10	0.12	0.10	0.07	0.10	0.08	0.10	0.10
S.E. of mean	0.015	0.019	0.016	0.013	0.016	0.011	0.014	0.016

observed are shown in Table IV. When these control and experimental groups are compared, no statistically significant differences between the growth rates of the groups of snails are found ($p = 0.3$, using the Kruskal-Wallis test).

DISCUSSION

The effect of 2B on the snail *Physa heterostropha* is quite different from that of the other drugs studied and is of a nature which indicates its action on the snail is not simply one of inhibition of carbonic anhydrase. This result agrees well with the conclusion of Wilbur and Jodrey (1955) that this drug has an effect on calcium deposition in the oyster that is independent of its inhibition of carbonic anhydrase and in their experiments was shown to be independent of the mantle.

The results of experiments using Diamox, BS, TS, and SA with snails fed for rapid growth are consistent with the hypothesis that rapid shell growth in this mollusc depends upon the activity of carbonic anhydrase and it is unlikely that such congruence of effects would occur if the four drugs were acting on different enzyme systems. At low growth rates induced by feeding method I, Diamox did not further decrease growth rates, indicating that under at least certain circumstances of slow growth carbonic anhydrase activity is insignificant in shell growth

of the snail. Such a result does not, however, preclude the possibility that the enzyme may have increased importance in determining the rate of growth under other circumstances limiting shell development, as low temperatures.

The results here reported are entirely consistent with the hypothesis that under certain circumstances carbonic anhydrase is essential for rapid shell development in the snail used but that in some conditions the enzyme is not necessary for shell growth.

Summary

1. The rate of growth of *Physa heterostropha* may be controlled in the laboratory by using different modes of feeding.

2. Under the conditions of the experiments, growth rates for each feeding method, with the possible exception of that giving slowest growth, are constant over the range 3.00 to 5.99 mm. median shell lengths during two-day growth periods but are slower at both higher and lower lengths.

3. The drug 2-benzothiazolesulfonamide completely inhibits growth of the snail at concentrations about 1 mg. per liter. At 21° C. it is lethal at concentrations above this but appears to be somewhat less toxic at 26° C. Its effect is not due to the action of the drug as a carbonic anhydrase inhibitor but to an unknown side effect.

4. Diamox, benzenesulfanilamide, p-toluenesulfonamide and sulfanilamide, at the concentrations exhibiting maximum effect on growth, exhibit comparable degrees of inhibition of shell growth in snails fed for rapid growth.

5. Diamox at concentrations up to 1.5 mg. per liter does not decrease the growth rate of snails induced to grow slowly by the feeding method employed though this concentration inhibited growth to a significant degree in rapidly growing snails.

6. The results are consistent with the hypothesis that carbonic anhydrase is essential in rapid shell development of some molluscs but may be insignificant in slow growth induced by poor feeding conditions, and afford additional evidence that the enzyme is significant in mollusc shell formation.

LITERATURE CITED

Abolins-Krogis, A., 1958. The morphological and chemical characteristics of organic crystals in the regenerating shell of *Helix pomatia* L. *Acta Zool.*, **39**: 19–38.

Berliner, R. W., and J. Orloff, 1956. Carbonic anhydrase inhibitors. *Pharmacol. Rev.*, **8**: 137–174.

Clark, A. M., 1957. The distribution of carbonic anhydrase in the earthworm and snail. *Australian J. Sci.*, **19**: 205–206.

Florkin, M., and P. de Marchin, 1941. Distribution de l'anhydrase dans les tissus de l'anodante. *Arch. Internat. Physiol.*, **51**: 130–132.

Freeman, J. A., and K. M. Wilbur, 1948. Carbonic anhydrase in molluscs. *Biol. Bull.*, **94**: 55–59.

Ikinago, K., 1954. The metabolism of the pearl oyster, *Pinctada martensii*—I. On the carbonic anhydrase. *Bull. Japanese Soc. Fisheries*, **19**: 925–928. (In Japanese with English summary.

Kawai, K., 1954a. Carbonic anhydrase in pearl oyster. I. Distribution and some properties of the enzyme. *Mem. Coll. Sci., Univ. Kyoto., Ser. B*, **21**: 39–44.

Kawai, K., 1954b. Carbonic anhydrase in the fresh-water mussel (*Hyriopsis schlegelii*) and physiological significance of the enzyme. *Japanese J. Mal.* **18**: 39–46. (In Japanese with English summary.)

KREBS, H. A., 1948. Inhibition of carbonic anhydrase by sulphonamides. *Biochem. J.*, **42**: 525–528.

KRUSKAL, W. H., AND W. A. WALLIS, 1952. Use of ranks in one-criterion variance analysis. *J. Amer. Statist. Assc.*, **47**: 583–621.

LARRANETA, M. G., AND F. PONZ, 1954. Alkaline phosphatase and carbonic anhydrase in some gastropods. *Rev. Espan. Fisiol.*, **10**: 225–234.

MAETZ, J., 1946. L'Activité anhydrasique de quelques tissus d'Invertébrés. *Bull. Inst. Oceanogr.*, **43**: 1–20.

MANN, H. B., AND D. R. WHITNEY, 1947. On a test of whether one of two random variables is stochastically larger than the other. *Ann. Math. Statist.*, **18**: 50–60.

MANN, T., AND D. KEILIN, 1940. Sulphanilamide as a specific inhibitor of carbonic anhydrase. *Nature*, **146**: 164–165.

MELDRUM, N. U., AND F. J. W. ROUGHTON, 1933. Carbonic anhydrase. Its preparation and properties. *J. Physiol.*, **80**: 113–170.

MILLER, W. H., A. A. DESSERT AND R. O. ROBLIN, JR., 1950. Heterocyclic sulfonamides as carbonic anhydrase inhibitors. *J. Amer. Chem. Soc.*, **72**: 4894–4896.

STOLKOWSKI, J., 1951. Essai sur le déterminisme des formes minérologiques du calcaire ches les êtres vivants (calcaires coquilliers). *Ann. Inst. Oceanogr.*, **26**: 1–113.

TSUJII, T., AND A. MACHIDA, 1953. Carbonic anhydrase and the formation of pearls. *Kagaku*, **23**: 148.

WILBUR, K. M., AND N. G. ANDERSON, 1950. Carbonic anhydrase and growth in oyster and Busycon. *Biol. Bull.*, **98**: 19–24.

WILBUR, K. M., AND L. H. JODREY, 1955. Studies on shell formation. V. The inhibition of shell formation by carbonic anhydrase inhibitors. *Biol. Bull.*, **108**: 359–365.